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The new PFAS C6O4 and its effects on marine invertebrates: First evidence of transcriptional and microbiota changes in the Manila clam Ruditapes philippinarum

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ABSTRACT

There is growing concern for the wide use of perfluorooctanoic acid (PFOA) because of its toxic effects on the environment and on human health. A new compound – the so called C6O4 (perfluoro ([5-methoxy-1,3-dioxolan-4-yl]oxy) acetic acid) - was recently introduced as one of the alternative to traditional PFOA, however this was done without any scientific evidence of the effects of C6O4 when dispersed into the environment. Recently, the Regional Agency for the Protection of the Environment of Veneto (Italy) detected high levels of C6O4 in groundwater and in the Po river, increasing the alarm for the potential effects of this chemical into the natural environment. The present study investigates for the first time the effects of C6O4 on the Manila clam *Ruditapes philippinarum* exposed to environmental realistic concentrations of C6O4 (0.1 μg/L and 1 μg/L) for 7 and 21 days. Furthermore, in order to better understand if C6O4 is a valid and less hazardous alternative to its substitute, microbial and transcriptomic alterations were also investigated in clams exposed to 1 μg/L of PFOA. Results indicate that C6O4 may cause significant perturbations to the digestive gland microbiota, likely determining the impairment of host physiological homeostasis. Despite chemical analyses suggest a 5 times lower accumulation potential of C604 as compared to PFOA in clam soft tissues, transcriptional analyses reveal several alterations of gene expression profile. A large part of the altered pathways, including immune response, apoptosis regulation, nervous system development, lipid metabolism and cell membrane is the same in C6O4 and PFOA exposed clams. In addition, clams exposed to C6O4 showed dose-dependent responses as well as possible narcotic or neurotoxic effects and reduced activation of genes involved in xenobiotic metabolism. Overall, the present study suggests that the potential risks for marine organism following environmental contamination are not reduced by replacing PFOA with C6O4. In addition, the detection of both C6O4 and PFOA into tissues of clams inhabiting the Lagoon of Venice - where there are no point sources of either compounds - recommends a similar capacity to spread throughout the environment. These results prompt the urgent need to re-evaluate the use of C6O4 as it may represent not only an environmental hazard but also a potential risk for human health.

1. Introduction

Per- and poly-fluoroalkyl substances (PFAS), such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), have been found in surface and groundwater worldwide and detected globally in the tissues of fish, bird, and marine mammals (Fujii et al. 2007). They result from

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manufacturing processes (Buck et al., 2011) and due to their extraordinary persistence, bioaccumulation tendencies and toxicological effects, PFAS represent an intrinsic threat for organisms and human health (EPA, 2017; Zheng et al., 2019). Following the industrial phase-out of PFOA, perfluoro([5-methoxy-1,3-dioxolan-4-yl]oxy) acetic acid ($C_6HF_9O_6$), commercially known as C6O4 or F-Diox acid, has been recently introduced as one of the alternative substances. C6O4, a short-chain perfluoropolyether substance, has been registered and patented by Solvay in 2014 to replace PFOA in accordance with the international EPA-PFOA Stewardship Program that proposed the abolition of PFOA and related chemicals by 2015 (ECHA, 2019; EFSA, 2014; Guruge et al., 2006; Kjølholt et al., 2015; Lu et al., 2019). The purpose was to reduce and possibly abolish environmental contamination as well as eliminating any risk for human health from PFOA, which is recognized as a major problem worldwide (EPA 2017; Bonato et al. 2020). All the chemical forms of C6O4 (i.e. acid, ammonium and potassium salt) are highly soluble and completely dissociate in aqueous solutions, and, due to the strong C-F bond, they seem to be resistant to biodegradation in water (ECHA, 2019).

Even though C6O4 is constantly detected in aquatic habitats (Morganti et al., submitted for publication; ARPAV, 2020, 2019), no environmental limits have been established yet, probably also due to a crucial lack of information about its ecotoxicological effects. Publications from the Stockholm Convention stated that new generation of fluorinated substances (NGPFASs) with a short carbon chain (i.e. C4- and C6-) can replace long-chain ($C \geq 8$) homologues because potentially less hazardous, accumulative, and toxic (Chappell et al., 2020; Chen et al., 2018a; Kjølholt et al., 2015). In general, compared with long-chain PFAS, the short chain perfluoropolyether substances (e.g. GEN-X; ADONA) seem to be able to increase the solubility and the elimination speed and also appear to have a lower affinity for critical receptors in living organisms (Liu et al., 2020). However, recent studies demonstrated that these chemicals are persistent and detected in both abiotic and biotic environments (Strynar et al., 2015; Washington et al. 2020, Pan et al. 2018). Overall, while a large amount of studies confirms that finding valid substitutes to long-chain fluorinated compounds is extremely urgent due to their deleterious effects at molecular, cellular, and physiological levels (Wei et al., 2009; Liu et al., 2020), knowledge about the potential effects of NGPFAs in living organisms is still extremely scarce.

To date, the possible toxicity of C6O4 has been assessed only in the dossier of the European Chemicals Agency for REACH regulation, which determined the EC50 in *Daphnia magna* and *Danio rerio* respectively exposed to C6O4 for 48 and 96 h, via ecotoxicological tests (ECHA, 2019). Given the fact that C6O4 is increasingly employed in a wide range of industrial and consumer applications, knowledge of the potential effects of C6O4 in marine organisms assumes a crucial importance to support the management and the prospective political decisions.

In this context, a recent monitoring program carried out by the Regional Environmental Protection Agency of Veneto (Italy) detected relatively high levels of C6O4, up to 3200 ng/L in ground-water and about 300 ng/L in the Po river (northeast of Italy) (ARPAV, 2020, 2019). Moreover, another study revealed the presence of C6O4 in the surface water and eggs of wild bird collected nearby the Solvay production area of Spinetta Marengo (Alessandria, Piedmont, Italy) (Morganti et al., submitted for publication). These data have greatly increased public awareness and concern for this chemical, and generated a heated public debate on the real environmental and health risks associated with C6O4. However other anthropogenic sources, such as the release from various industrial and commercial materials (e.g. plastic caps, products of thermoplastic industry such as sealants resistant to heat and acids) and environmental release through waste water treatment plants and atmospheric precipitation cannot be excluded in high industrialized and populated areas.

The present study reports for the first time on the effects of C6O4 on a marine invertebrate, the Manila clam *Ruditapes philippinarum*. To reach such a goal, changes in transcriptional response, bioaccumulation, and microbial communities were investigated in clams exposed for 7 and 21 days to environmentally realistic concentrations of C6O4 (0.1 μg/L and 1 μg/L). Furthermore, in order to better understand whether C6O4 is a valid and less hazardous substitute of PFOA, the same interdisciplinary approach has been applied in clams exposed to 1 μg/L of PFOA.

The Manila clam is an ecologically and commercially relevant edible bivalve species that inhabits lagoons and river deltas. Its choice in this study has twofold value as it is well studied as sentinel of environmental stress (Claus et al., 2016; Milan et al., 2018) and, at the same time, it represents the most important clam species cultured in Italy and the EU for human consumption.

2. Material and methods

2.1. Experimental design

Manila clams (3.64 ± 0.32 cm shell length) were collected within the Venice lagoon in February 2020. Before experiments, bivalves were acclimated in the laboratory for one week in large aquaria with aerated natural seawater (salinity of 35 ± 1‰, temperature of 12 ± 0.5 °C), previously transported from the coast to the laboratory, and were fed daily *ad libitum* with microalgae (*Isochrysis galbana*). Only healthy animals, namely those showing reborrowing capability and extension of syphons, were used for the experiments. Duplicate glass tanks were used for each exposure concentration as well as for control animals. A total of 45 clams were maintained in each tank (90 animals for each experimental group).

Perfluoro([5-methoxy-1,3-dioxolan-4-yl]oxy) acetic acid (C6O4) (CAS no. 1190931-41-9) and PFOA (CAS no. 335–67-1) were added at different concentrations, namely low (L) and high (H): 0.1 μg/L C6O4 (named C6O4_L), 1 μg/L C6O4 (named C6O4_H) and 1 μg/L PFOA (named PFOA_H) and maintained for 21 days. As the stock solution of C6O4 was prepared in methanol, in the control group (named CTRL) methanol was added at the highest concentration (20 μL/L) used in the C6O4 treatments (1 μg C6O4/L). Seawater and contaminant concentrations were renewed every 48 h. During exposure, clams were fed every 48 h by adding 200 mL ($6 * 10^5$ cell/mL) of microalgae solution per each tank. Samples of each experimental group were collected after 7 and 21 days of contaminants' exposure and used for subsequent analysis. Non-significant mortality of samples was observed in any group during the exposure period.

2.2. Chemical analyses

C6O4 and PFOA concentrations were measured three times in water samples collected from the exposure tanks: approximately 15 min after C6O4 and PFOA addition (t = 0), after 24 h and 48 h (respectively before water renewal and second application of C6O4 and PFOA). Seawater samples (50 mL per tank) were collected and stored at −20 °C. C6O4 and PFOA quantification in water matrices was performed by using the ISTISAN method ISS.CBA.052.REV00, developed by Istituto Superiore di Sanità (ISS) for drinking water analysis. Seawater samples were centrifuged (1 cycle at 3000 g) prior to analysis and then diluted 1:50 due to high concentration of dissolved NaCl. Samples were collected into vials, ready for direct injection into LC-MS system (ESI-). The analyses were performed using a TQ6500 mass spectrometer (Sciex, Canada) coupled with a UPLC Nexera-X2 system (Shimadzu, Kyoto, Japan). Analyses of C6O4 and PFOA bioaccumulation was carried out on soft tissue samples (10 specimens per concentration) and haemolymph (10 specimens per concentration) from clams exposed for 21 days. Haemolymph was collected from the anterior adductor muscle by a 1-mL plastic syringe, placed in Eppendorf tubes and stored on ice. Thereafter, soft tissues were excised, stored on ice, and then frozen in liquid nitrogen (along with haemolymph samples) and stored at −80 °C until processing. The

extraction of C6O4 and PFOA was carried out according to the method of Mazzoni et al. (2016). Briefly, samples (about 10 g of clam soft tissue and about 3.5 g of haemolymph) were spiked with 100 μL of an internal standard methanolic solution (40 μg/L, MPFAC-MXA and M3PFPeA solutions, Wellington Laboratories, Guelph, ON, Canada) and a mixture of acidified water-acetonitrile (10:90 v/v) solution was added to the spiked sample. The samples were subjected to ultra-sonication extraction, a treatment with $MgSO_4$/NaCl, freezing and centrifugation. Afterwards, the extracts were partially evaporated and then filtered by HybridSPE®-Phospholipid Ultra cartridges (Merck KGaA, Darmstadt, Germany) to eliminate phospholipids. The final extracts were analyzed by liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS) after an online purification with turbulent flow chromatography (TFC). Quantification was done using the isotopic dilution method. Limits of Detection (LODs) and Limits of Quantification (LOQs) were estimated, according to ISO Standard 6107-2: 2006, as respectively, three-fold and tenfold the standard deviation of a procedural blank. LOD and LOQ values for PFOA were 0.02 and 0.08 μg/kg ww for clam soft tissue samples and 0.07 and 0.27 μg/kg ww for haemolymph samples. LOD and LOQ values for C6O4 were 0.04 and 0.16 μg/kg ww for clam soft tissue samples and 0.14 and 0.54 μg/kg ww for haemolymph samples.

2.3. RNA extraction and preparation of libraries

After 7 days and 21 days, the digestive gland of 20 specimens from each experimental group was collected and stored in RNA later at −80 °C until further use. For each experimental group (CTRL, C6O4_L, C6O4_H and PFOA_H) and sampling time (Day 7 and Day 21), 5 pools, each composed by 4 digestive glands, were prepared for total RNA extraction (RNeasy Mini Kit Qiagen, Hilden, Germany). RNA purity, concentration, and integrity of each pool were checked using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and Tape Station (Agilent, Waldbronn, Germany). RNA extracted from each pool was used for both gene expression (RNA-Seq) and microbiota analysis (16S). Library preparation for gene expression analysis was performed using Illumina TruSeq RNA Library Prep Kit. The library pools were sequenced on Illumina Novaseq 6000 (CRIBI; University of Padova) with a paired-end 2x100 bp setup obtaining a total of 1,128,494,641 paired reads (sequences available in NCBI SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA663745). For microbiota characterization, 1 μg of RNA was reverse-transcribed to cDNA using the Superscript IV kit (Invitrogen, Life Technologies, Monza, Italy). Libraries and sequencing were performed by BMR Genomics (Padova, Italy) in a 50 μL reaction starting with diluted 0.2 ng/μL cDNA and both reverse and forward primers (10 μM) that specifically target the V3–V4 gene region of the bacterial 16S rRNA as described by Milan et al. (2018). The final libraries were then sequenced with MiSeq Illumina 300 PE. Microbiome sequencing generated 2,462,724 reads (sequences available in NCBI Sequence Read Archive SRA https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA663745).

2.4. Microbiome data analysis

Raw sequencing data were analysed using QIIME2 (Quantitative insights into microbial ecology 2; Bolyen et al., 2019). Reads were trimmed, filtered, and merged with cutadapt and DADA2 respectively to exclude primer sequences and, based on the nucleotide assignment, reads with quality lower than 20 (Phred Score). Features were aligned using MAFFT software (Katoh and Standley, 2013). After the quality-filter step, chimeric amplified fragments were removed and sequences merged to a total of 1,889,481 reads. Merged reads were classified with Python library scikit-learn and taxa assignment was performed using a pre-trained SILVA-database (Yilmaz et al., 2013). Final feature table and relative taxonomy were exported and analysed using CALYPSO (version 8.84) comparing all treated groups with their relative controls. In detail, to investigate changes occurring in microbial composition between CTRL and exposed clams, one-way ANOVA was carried out within each sampling time at OTU (operational taxonomic unit) and genus levels. PCoA at species level were also performed on the whole dataset as well as within each sampling time (7 and 21 days) using CALYPSO. Functional analysis of detected microbial communities was performed through PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software (Langille et al. 2013). STAMP software was used to graphically represent the obtained results.

2.5. Gene expression analyses

Gene expression profiles were explored through three different approaches: i) principal component analysis (PCA) as unsupervised method; *ii)* pairwise comparisons between CTRL and exposed clams (C6O4_L; C6O4_H; PFOA_H) at each sampling point (day 7 and day 21) followed by enrichment analyses; *iii)* GSEA focused on the pathways and biological processes found differentially regulated in response to chemical exposures and/or correlated to chemical concentrations in previous studies (e.g. Milan et al. 2013a, 2015; Iori et al. 2020).

In detail, FastQC/v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to quality check raw reads, which were subsequently trimmed to remove adaptors and low-quality reads using Trimmomatic/v0.36 (Bolger et al., 2014) with default parameters. High-quality reads were subsequently mapped against the reference transcriptome from the digestive gland, by using Kallisto/v0.46.1 (Bray et al. 2016) with default settings. The count table was generated using the "abundance_estimates_to_matrix.pl" script from the Trinity suite (Haas et al. 2013). Raw read counts were then imported into R/v3.6.0 (R Core Team 2014) and filtered: we removed contigs with less than 5 reads in at least 24 libraries (out of 40) which would contribute to background noise (Peruzza et al. 2020, Pradhan et al. 2020). Filtered reads were then normalized using the RUVs function (with parameter "k = 9") from the RUVSeq/v1.18 library (Gerstner et al. 2016; Verma et al. 2020) and then normalized counts were used to perform pairwise comparisons with edgeR/v3.26.0 (Robinson et al., 2010) comparing each of the treated groups (i.e. C6O4_L, C6O4_H and PFOA_H) against the control group (i.e. CTRL) at the same time point (i.e. day_7 or day_21). Genes with FDR < 0.05 and FC ≥ 2 were deemed differentially expressed.

Functional annotation of the reference transcriptome was performed by Blastx similarity search on Swissprot (Uniprot), *Homo sapiens* protein Ensembl database, *Danio rerio* protein Ensembl database and *Crassostrea gigas* protein Ensembl database (*Evalue* < 0.0001). Of 53,476 unique sequences, 32,711 (61%) showed at least one significant match. Details and the annotation of each contig is reported in Iannello et al. (submitted for publication). Zebrafish Ensembl IDs matching differentially expressed contigs were then used to build Venn diagrams at each sampling point.

Using the SwissProt IDs to match our contig IDs, enrichment analysis was performed on differentially expressed genes using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Dennis et al., 2003; Huang et al., 2008) and considering Gene Ontology Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) databases, and KEGG pathways (KP) database. To investigate enriched GO and KP between our groups, DAVID analysis was performed considering each entire list of differentially expressed genes, as well as considering up- and down-regulated genes separately, with the following settings, gene count 2 and ease 0.05.

For each pairwise comparison, we further performed Gene Set Enrichment Analysis (GSEA) (Subramanian et al. 2005) to reveal coordinated changes in gene expression within all genes. GSEA was performed using the R package clusterProfiler/v3.12.0 (Yu et al., 2012) using custom gene sets downloaded from https://www.gsea-msigdb.org/gsea/index.jsp. Gene sets with FDR < 0.1 were deemed statistically significant.

3. Results

3.1. Chemical analyses

The concentrations of C6O4 and PFOA measured in the experimental tanks and in exposed clams are reported in Supplementary file S1 and summarized in Table 1. During the exposure period, no loss of spiked chemicals was observed. To calculate bioaccumulation factors (BAF) in clams, the mean concentration of each chemical measured in the water was used. Concentrations in soft tissue of control specimens were 1.5 and 0.22 μg/kg ww for PFOA and C6O4, respectively and may give an indication of the background concentrations in soft tissues in the collection area.

Clams accumulated both C6O4 and PFOA, but at different proportion depending on the tissue: in soft tissues PFOA is accumulated more than five times than C6O4 (BAF = 119 and 21 in PFOA_H and C6O4_H, respectively), while in the haemolymph the accumulation of PFOA is only 0.6 times the accumulation of C6O4 (5.2 and 9.5 μg/kg ww for PFOA and C6O4 respectively, Table 1).

3.2. Microbiota characterization

Clam-associated bacterial communities consisted primarily of *Alphaproteobacteria* (51–70%), *Gammaproteobacteria* (16–39%) and *Chlamydiae* (1–11%). The overall distribution of classes for each sample group is reported in Supplementary Table S2. The PCoA analysis showed a clear separation along the x-axis (PC1 = 38% of variability) between clams collected at 7 and 21 days (Fig. 1A). Within each sampling time, a weak separation at 7 days along the x-axis between treatments has been observed (22% of variability; Fig. 1C), while clams exposed to C6O4_L were clearly separated from all other treatments at 21 days (Fig. 1B and D). A trend of higher microbial species richness and evenness was detected across all C6O4 treatments at 21 days compared to 7 days, while no significant differences were found between control and exposed groups at both sampling times (Fig. 1E and 1F).

The full lists of significant OTUs and genera obtained for each comparison between CTRL and exposed clams are reported in Supplementary Table S3 and summarized in Table 2. Unexpectedly, results showed the highest number of significantly differentially represented OTUs and genera in response to the lower C6O4 concentration (C6O4_L), confirming the results obtained by PCoA (Table 2). In detail, the comparison between CTRL and C6O4_L revealed a total of 13 and 68 significant OTUs after 7 and 21 days, respectively. Noteworthy, at 7 days clams exposed to C6O4_L showed a significant over-representation of genera *Vibrio* and *Arcobacter*, while an opposite trend has been observed for *Methylophaga,* which found down-represented in exposed clams (FDR < 0.05; see Supplementary file S3). At 21 days, C6O4_L showed several significantly over-represented OTUs matching the genera *Methylotenera* and *Polaribacter*. Clams exposed to the highest C6O4 concentration (C6O4_H) showed weaker changes in comparison to C6O4_L. However, the differentially represented taxa were similar to those detected at the lowest C6O4 concentration (i.e. *Polaribacter*, *Vibrio* spp. and *Methylophaga*). In addition, the genera *Arcobacter* and *Vibrio* were almost significantly over-represented at 7 days in C6O4_H exposed clams (p-value < 0.05; FDR = 0.1), confirming similar microbial dynamics to those detected in C6O4_L exposed clams. Clams exposed to PFOA_H showed similar microbial profiles to those detected in C6O4_H clams, with genera *Polaribacter* and *Methylophaga* respectively over- and under-represented at 7 days (FDR < 0.05). At the same sampling time, the over representation of the genera *Vibrio* (p-value < 0.05) and *Methylotenera* should also be highlighted. To conclude, at 21 days all treatments led to the down-representation of the genera *Profundimonas* and *Algicola* (p-value < 0.05; Table 2).

To investigate more thoroughly the functional diversity of bacterial communities between control and exposed clams, the software PICRUST was used. The differences identified in each treatment in KP functional categories are reported in Supplementary Figure S1. Overall, analyses of the KP in exposed groups revealed changes in several microbial metabolic functions, among which “fatty acid biosynthesis” (C6O4_L at day 21 and PFOA_H at day 7), “fatty acid metabolism” (PFOA_H at day 7), “synthesis and degradation of ketone bodies” (C6O4_L at day 21 day), and “benzoate degradation” (PFOA_H at both sampling time). Noteworthy, a significant decrease in “butanoate metabolism” was found in clams exposed to both C6O4 concentrations. The disruption of several pathways involved in the metabolism of amino acids was found in all treatments, such as “Valine, Leucine and isoleucine degradation” (in C6O4_L), “Lysine degradation” (in C6O4_L and PFOA), “Lysine biosynthesis” (in PFOA_H), “Tryptophane metabolism” (all treatments), and “Tyrosine metabolism” (in PFOA).

3.3. Gene expression analyses

PCA revealed a good discrimination between treatments at each sampling point (Fig. 2). After 7 days clams exposed to C6O4_H and C6O4_L clustered together, while a separation along the x- and y-axis was evident for PFOA_H exposed clams. Noteworthy, after 21 days PCA revealed a similar trend to those obtained by PCoA applied to clam’s microbiota (Fig. 1D), with a clear separation along the x-axis of clams exposed to the lower C6O4_L concentration, while along the y-axis PFOA_H and C6O4_H exposed clams appeared separated from the CTRL group.

Pairwise comparisons between control and exposed clams at 7 days showed the highest number of differentially expressed genes (DEGs) in response to PFOA_H (685 DEGs) (Table 3). At 21 days the highest number of DEGs was found in clams exposed to the lowest C6O4 concentration (C6O4_L; i.e. 501 DEGs), while transcriptional changes in response to C6O4_H and PFOA_H decreased (475 and 339 DEGs, respectively). It should also be highlighted that, at 21 days, all treatments showed a majority of down-regulated genes compared to CTRL, with an opposite trend to that observed at day 7 (Table 3). The full lists of DEGs are reported in Supplementary Table S4. Venn diagrams were then constructed considering *Danio rerio* gene IDs (see methods) to

Table 1
PFOA e C6O4 concentrations in exposure water and in the soft tissue and haemolymph of Manila clams after 21 days of exposition.

Sample	Water nominal concentration (μg/L)	Water measured concentration (μg/L)	Sample (g ww)	Sample (mL)	PFOA (μg/kg ww)	C6O4 (μg/kg ww)	Bioaccumulation Factor (L/kg)
Clam soft tissue							
CTRL	0	<LOD	15.51		1.5	0.22	
C6O4_L	0.1	0.11 ± 0.02	24.41		0.73	2.3	20
C6O4_H	1	1.01 ± 0.07	19.41		0.58	21.1	21
PFOA_H	1	0.93 ± 0.31	24.84		110	0.18	119
Clam haemolymph							
CTRL	0	<LOD	3.51	3	0.30	<0.14	
C6O4_L	0.1	0.11 ± 0.02	3.74	3.6	<0.07	<0.14	
C6O4_H	1	1.01 ± 0.07	4.18	3.6	<0.07	9.5	
PFOA_H	1	0.93 ± 0.31	2.91	2.5	5.2	<0.14	

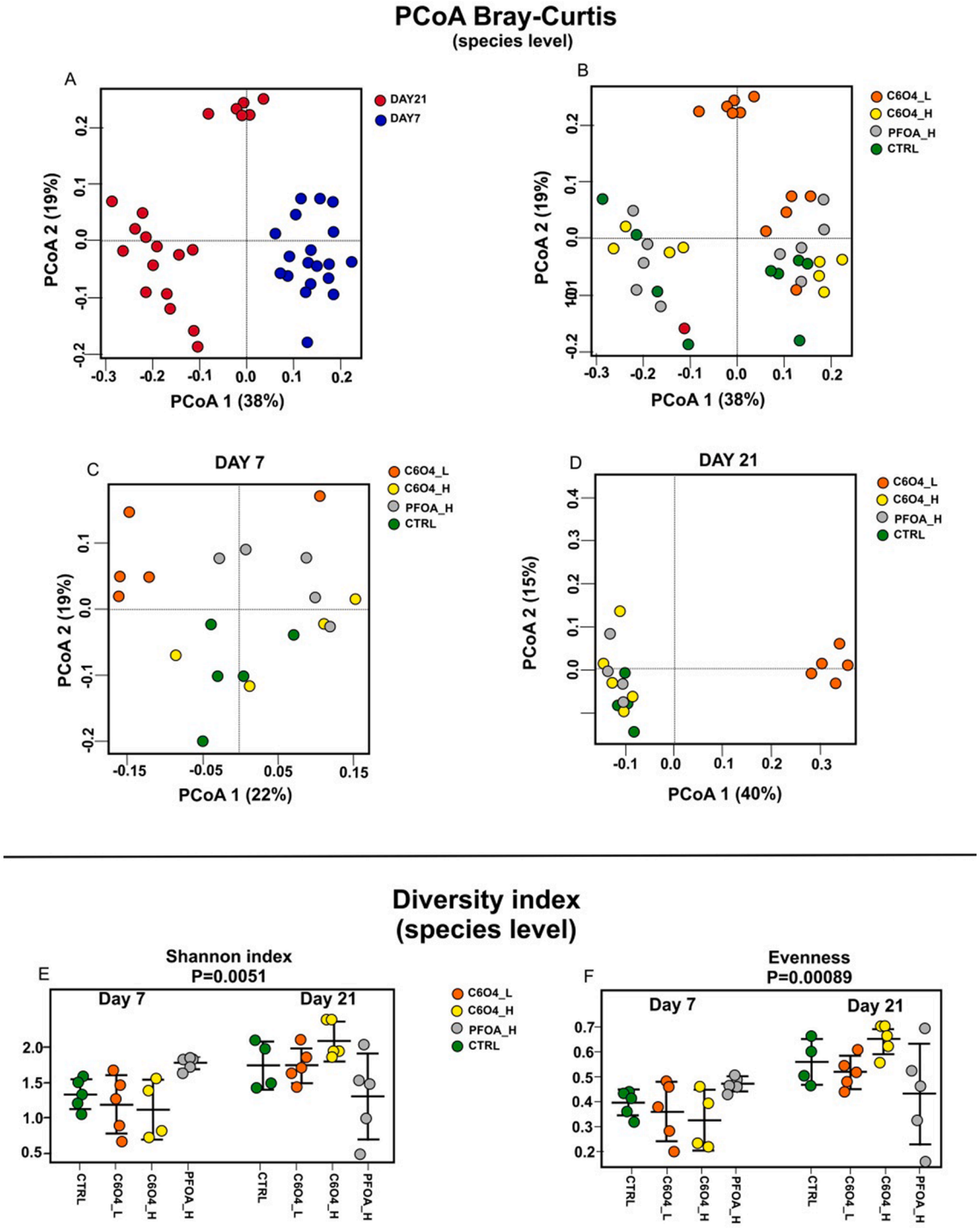

Fig. 1. Principal Coordinates Analysis (PCoA) of clam digestive gland microbiota. (A) Different colors indicate the two sampling time (Day 7 and Day 21); (B–D) Different colors indicate different treatment considering the whole experiment duration (B), and each sampling time separately (C and D); (E and F) Richness diversity index (Shannon index) and evenness for each group at 7 and 21 days.

define the gene list of DEGs for each treatment/sampling point (Fig. 3). Venn diagrams showed a total of 77 and 41 common DEGs shared between all three treatments at 7 and 21 days, respectively. Clams exposed to the two C6O4 concentrations shared 61% and 36% of DEGs at 7 and 21 days, respectively. It is worth to note that 61.7% (at 7 days) and 48.3% (at 21 days) of DEGs detected in response to C6O4_H were in common with those identified in response to PFOA_H at the correspondent sampling times. Similarly, clams exposed to the lowest C6O4 concentration shared with PFOA_H exposure 57.1% and 39.1% of DEGs at 7 and 21 days, respectively.

The results obtained by the enrichment analyses are summarized in Table 3, while the full lists of enriched terms are reported in Supplementary File S5. Among the shared responses across treatments, several genes involved in "nervous system development" were commonly found differentially expressed in all treatments/sampling times (Fig. 3). These results were confirmed in DAVID by the enrichment of the term "central nervous system development" (Table 3 and Supplementary File S5). In addition, the enrichment of the "Notch signalling pathway" was

Table 2
Number of significant taxa obtained through one way ANOVA analyses at OTUs and genus level comparing control group to groups exposed to C6O4 and PFOA at each sampling time (FDR < 0.05). The significant taxa showing similar trend among treatments are also listed (p-value < 0.05; in bolt significant taxa with FDR < 0.05). The full lists of significant taxa at OTUs and genus level with corresponding p-value and FDR are reported in Supplementary file S3.

	OTU level (FDR<0.05)					
	Day 7			Day 21		
	N° Sign. OTUs	N° ↑ OTUs	N° ↓ OTUs	N° Sign. OTUs	N° ↑ OTUs	N° ↓ OTUs
C6O4_L	13	6	7	68	48	20
C6O4_H	7	5	2	2	1	1
PFOA_H	7	4	3	4	0	4
	Genus level (FDR<0.05)					
	Day 7			Day 21		
	N° Sign. Genus	N° ↑ Genus	N° ↓ Genus	N° Sign. Genus	N° ↑ Genus	N° ↓ Genus
C6O4_L	4	2	2	23	17	6
C6O4_H	4	2	2	0	0	0
PFOA_H	4	3	1	0	0	0

Significant taxa (p-value<0.05)			
	C6O4_L	C6O4_H	PFOA_H
Day 7	***Vibrio*** ***Arcobacter*** *Polaribacter_1* ***Methylophaga***	***Polaribacter*** *Vibrio* *Arcobacter* ***Methylophaga***	***Polaribacter*** ***Methylotenera*** *Vibrio* ***Methylophaga***
Day 21	***Polaribacter_1*** ***Polaribacter_3*** ***Methylotenera*** ***Profundimonas*** ***Algicola***	***Vibrio_otu*** *Profundimonas* *Algicola*	*Profundimonas* *Algicola*

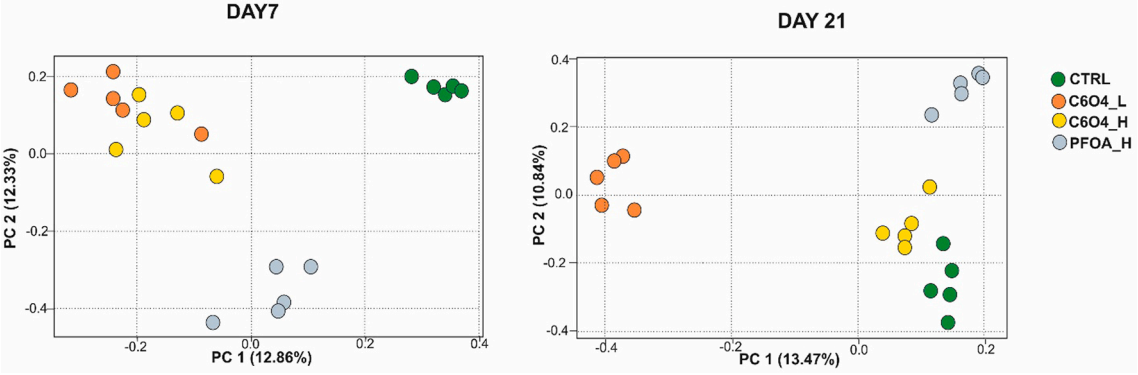

Fig. 2. PCA applied to gene expression profiles at each sampling time.

detected in clams exposed to PFOA_H and C6O4_L, including several putative notch receptors (*notc1*, *notc2*, *notc3*; Table 3). Similarly, several genes encoding members of the slit family (*slit1*, *slit2*, *slit3*), a family of secreted extracellular matrix proteins that play an important signalling role in neural development, were up- and down-regulated in response to both C6O4 and PFOA exposure after 7 and 21 days, respectively (Supplementary File S4).

At both sampling times, several enriched biological processes (BP) and pathways related to immune response were found modified in all treatments. In details, up-regulation of several genes involved in immune response, such as coding for C-type lectin (*clc4a*; *clc10*; *mrc1*), complement C1q-like proteins (*c1qt3*, *c1ql3*; *co5*, *c1*), interferon-inducible GTPase 1 (*iigp1*), soluble scavenger receptor cysteine-rich domain-containing protein SSC5D (*srcrl*), was observed after 7 days

Table 3
The upper part of the table reports the number of differentially expressed genes obtained comparing the control group to groups exposed to C6O4 and PFOA at each sampling time. Some of the enriched terms detected in response to each condition/sampling point are also reported indicating the DEGs involved in each biological processes/KEGG pathways. Genes resulted over- and under-transcribed in clams exposed to each treatment are indicated in red and green, respectively (in black genes represented by more than one significant contigs showing opposite response. The full lists of DEGs and enriched terms are reported in Supplementary file S4 and Supplementary file S5.

	D7			D21		
	N° DEG	N° ↑	N° ↓	N° DEG	N° ↑	N° ↓
C6O4_L	579	446	133	501	247	254
C6O4_H	619	561	58	339	116	223
PFOA_H	685	500	185	475	113	362

Enriched terms/functions	C6O4_L		C6O4_H		PFOA_H	
	Day 7	Day 21	Day 7	Day 21	Day 7	Day 21
Enriched Biological Process / KEGG pathway						
Immune response/Inflammation						
Immune response/Innate immune response	TRAF3; DIAP2; VNN1; TCAM2; CO5; IIGP1; CLC4A; SRCRL; PGSC2; TIF1B	TNF15; IF44L; SAMH1; CO9; TGTP2;	CLC10; STING; DIAP2; VNN1; CO5; IIGP1; TIF1B; CLC4A; SRCRL; PGSC2	TNF15; TSP1; PXDN; IF44L; TGTP2	CLC10; TCAM2; SAMH1; IIGP1; SRCRL; CD209; DIAP2; TRAF3; SAMH1; VNN1; TIF1B; TIF1B; YM67	IF44L; TSP1; TGTP2; SAMH1
Defense response to virus	TCAM2; GBP1, IF44L; GBP3	IF44L; DMBT1; SAMH1; FCN3			SAMH1; TCAM2; GBP3, IF44L	IF44L; GBP1; SAMH1;
Cell adhesion	TENA; PTPRK; BGH3; LYAM2; PGCA; POSTN; SVEP1; CELR3; PTPRU; LYAM1; SDK1; FAT1; NPHN; SVEP1; FAT4	SVEP1; PTPRF; CELR1; MMRN1; NCAN; NPHN; MAG; TENX; PGCB;	FAT4; TENA; PTPRK; PGCA; BGH3; POSTN; SVEP1; NCAN; COCA1; LYAM1; FAT1; PGCB; PTPRT; NPHN; SVEP1; CO6A5; ITA4; SVEP1; HCK;	PTPRF; EMIL1; MMRN1; PGCB; PTPRU; CELR1; SVEP1; TSP1; DSCAM; NLGN1; TENA; CSPG2; FAT4; FAT1	PTPRK; BGH3; LYAM2; SVEP1; CNTN6; PGCB; CD209; TENM; PTPRT; NPHN; SVEP1; EMIL2; CNTN4; SVEP1; ALS; CELR3; SREC; COCA1; SDK1; DSCAM	SVEP1; PTPRF; PGCB; PTPRT; MMRN1; DSCAM; TSP1; LAR; FAT4; NPHN; NOTCH; COIA1; TENR; TENX; CSPG2; TENA; LRFN3; PTPRU; FAT1; HMCN2; BGH3
Other genes involved in inflammation / immune response	C1QL3 EDAR; MRC1; BDEF; C1QL4; C1QT6;	BGBP; LGR4; C1QL3; C1QL4; CL17A; CLC4A; MRC1; STA5B; C1QT5; CLC10; DUOX1;; DUOX2; GIMAP7; IIGP5; IL6RB; LECM; LYS; MRC2; TNSF10	DMBT1; C1QL4; TNSF10; CP4FE; EDAR;	BGBP; C1QL3; BDEF; C1QL4; C1QT4; CLC4A; RO60; TRAF3; TIF1B; DTX3L; IFI27; LECH; MCLN1; C1QRF; CL17A; CLC10	MRC1; STING; IFI27; IIGP5;	C1QL3; C1QL4; IF44L; NOTC2; CL17A; BGBP; C1QL2; C1QT2; FCER2; GBP1; IIGP1; LECH; LECM; LGR4; MRC1; PAR14; TRAF3; GIMAP1; TNSF10
Apoptosis						
Apoptosis	XIAP; NTRK1; BIRC2;		BIRC2; NTRK1; XIAP; TNFSF10		TNFSF10; BIRC2; NTRK1; XIAP;	
Other genes involved in apoptosis regulation	EDAR; GA45G; DIAP2; BIR7B; TNF15; BIRC8	TNF15; GIMAP7; EDAR; GA45G; DNAS1; FGFR3; RELT;	DIAP2; EDAR; CARD6;	TNF15; TRAF3; IFI27; FGFR3; PAK2; RB	EDAR; DIAP2; IFI27; RELT; DIDO1; SCRIB;	BIR7B; DIAP1; FGFR3; GIMAP1; TRAF3
Xenobiotic metabolism						
Xenobiotic metabolic process					ST1A4; ST1B2; CYP2B10; ST1B1; GST3	ST1A4; ST1A1 ST1B1
Sulfotransferase activity	ST1C4; ST1C2; ST1B1		ST1B1 ST1C4		ST1C4; ST1A4; ST1B2; S1C2A; ST2A1; ST1B1	ST1A4; ST1A1; ST1C4; ST2A1; S1C2A
Other genes involved in xenobiotic metabolism	MRP4	CP4P3; ST1B1; GST1; CYP356; CP3AO		ST1B1; CP2J5; ST1C4; CP4FN	CHST15; CP2BJ; CP2BA; CP2B4; GSTP1	CP2BJ; CP2B2; GST1; CP4F6;

(continued on next page)

Table 3 (*continued*)

Protein turnover						
Lysosome	SLC17A5; NAGA; GLA; CTSL;		NAGA; GM2A ; GLA; CLTB; CTSL;		NAGA; SLC17A5; GM2A ; GLA; NPC2; CTSL	
Protein ubiquitination			R213B; DIAP2; TIF1A; TRI33; R213A; TIF1B; TRI33		TIF1B; R213B; TRAF3; DIAP2; TIF1A; TRI33; TIF1A; R213A; TRI33	TIF1A; TRI33; R213B; TRAF3; R213A; RN213A
Cell Membrane						
Plasma membrane	MDGA1; AGRF1; AL3B1; MRC1; CELR3; SDK1; TRPA1; CRYL1; CSMD3; EDAR; EPHA5; FAT1; FAT2; FAT4; FCER2; GBP1; GP157; LIFR; LPH; LYAM1; LYAM2; MRP4; NICA; NOTC2; NPHN; PKND; PTP10; PTPRT; PTPRU; SLIT2; SPTN1; TCAM2; TNF15; TRPV1; VNN1; ZNT2; ABCA1; CD109		FAT4; MDGA1; CAD89; STING; TEN1; PTPRT; SLIT2; CD63; LPH; LPH; MKS3; SPTN1; LYAM1; FCER2; PTP10; LIFR; NCAN; PKND; CLH1; ITA4; FAT1; NETR; NETR; GP157; FAT4; LRC8D; CLC10; LRC8A; UNC5B; MCLN1; PTPRT; VNN1; GBRT; TEN2; NPHN; EDAR; NICA		MRC1; S28A1; UNC9; GSTP1; TRFR; NETR; SDK1; NETR; SREC; MDGA1; CELR3; CAD23; NOTC1; PTPRT; TENM; PLPP1; SLIT2; CD63; SPTN1; LYAM2; PTP10; HINT1; EGFR; Y4629; SAMH1; SAMH1; CD109; DOK3; LRC8D; TCAM2; CLC10; LRC8A; CNTN4; MCLN3; MCLN1; PTPRT; VNN1; TNR19; TNR19; TEN2; NPHN; EDAR; CNTN6; CAD89; FAT2	NOTC2; LAR; FAT4; NETR; FCER2; NPHN; EPHA5; FGFR3; LGR5; NOTC2; ANK1; SAMH1; SAMH1; GBP1; AGRB2; NICA; PTP10; NETR; LRFN3; PTPRU; GLUCL; NOTCH; FAT1; VNN3; SO2B1; PTPRO; MGA; NOTC1; UNC9; FLNC; LGR6; MRC1; PKND; NETR; GP157; DRPR; PTPRT
Integral component of plasma membrane	MRC1; ABCA1; EPHA5; FAT; FAT1; FAT2; LGR4; LPH; NOTC2; PKND; PTPRK; PTPRU; TRPV1	LGR4; NPHN; MRP3; FGFR3; LRC32; CLCA4; NOTC2; MRC2; CELR1; CEL; MRC1; NRT; SOAT; TUTL		FGFR3; FAT1; NLGN1; LGR6; S17A5; SC5A6; LGR5; LGR4; DSCAM; CELR1; PTPRU		NOTC2; LGR4; FGFR3; EPHA5; FAT1; PTPRO; MRC1; PTPRU; LGR6; SO2B1; NOTC2; LGR5; DSCAM; PKND
Glycosphingolipid biosynthesis	NAGA; GLA;		NAGA; GLA;		NAGA; GLA;	
Nervous system development						
Central nervous system development		PGCB; RUNX1 NCAN; CELR1	PGCA; NCAN; PGCB; VNN1	CSPG2; PGCB; PTP10, CELR1	PTP10; PGCB; VNN1; CNTN6	CSPG2; VNN3; PTP10; ARSB; PGCB
Notch signaling pathway	FAT4; OFUT1; NICA; FAT4; NOTC2	NOTC3;; NOTC1; DLD;				NOTC3; NOTC1; NOTCH; NICA; NOTC2; FAT4

(Table 3). Conversely, after 21 days an opposite trend was observed in all treatments, with the majority of genes involved in immune responses found down-regulated (Table 3). "Cell adhesion" was the enriched BP term with the highest number of DEGs in all treatments and sampling times, including several common DEGs across treatments.

Among the shared enriched pathways, "apoptosis" deserves particular attention as this was found enriched in all treatments at 7 days. In particular, putative baculoviral IAP repeat containing 2 (*birc2*), X-linked inhibitor of apoptosis (*xiap*), death-associated inhibitor of apoptosis 2 (*diap2*) and tumor necrosis factor receptor superfamily member EDAR (*edar*) genes were up-regulated in all treatments (Table 3). In addition, at 7 days, clams exposed to the low C6O4 concentration showed also the up-regulation of growth arrest and DNA damage-inducible protein GADD45 gamma (*gadd45g*) and baculoviral IAP repeat-containing protein 7-B (*bir7b*) genes, while C6O4_H showed the up-regulation of putative caspase recruitment domain family member 6 (*card6*). Several genes involved in regulation of apoptosis, including *gadd45*, tumor necrosis factor ligand superfamily member 15 (*tnf15*) and *edar* were down-regulated at 21 days in C6O4_L, showing an opposite regulation in comparison with the 7-days sampling time (Table 3).

In relation to protein turnover, the KP "Lysosome" was enriched in all treatments at 7 days, while the BP term "Protein ubiquitination" was enriched just in response to C6O4_H and PFOA_H at 7 days, in consequence of several up-regulated genes coding for E3 ubiquitin-protein ligases.

The enrichment of the BP "xenobiotic metabolic process" at both PFOA_H sampling times and the up-regulation of several genes coding for Cytochrome P450 (*cyp2b19*, *cyp2j5*, *cyp2b10*, *cyp2b20*, *cyp2b4*, *cypiva2*) suggest a much stronger response toward xenobiotic metabolism in PFOA exposed clams as compared to C6O4 exposed clams. However, putative sulfotransferases (*st1c4*, *st1c2*, *st1b1*) involved in sulfotransferase activity were up-regulated also in response to both C6O4 treatments at 7 days (Table 3).

Among pathways putatively involved in energy metabolism, the enriched BP term "positive regulation of adiponectin secretion" was found in all treatments and sampling times, while the BP term "negative regulation of gluconeogenesis" including putative Adiponectin (*adipo*) was enriched at both sampling times in C6O4_L and in response to PFOA_H at 21 days (Supplementary File S4 and S5). Venn diagram reported in Fig. 3, showed several DEGs involved in "Lipid metabolic

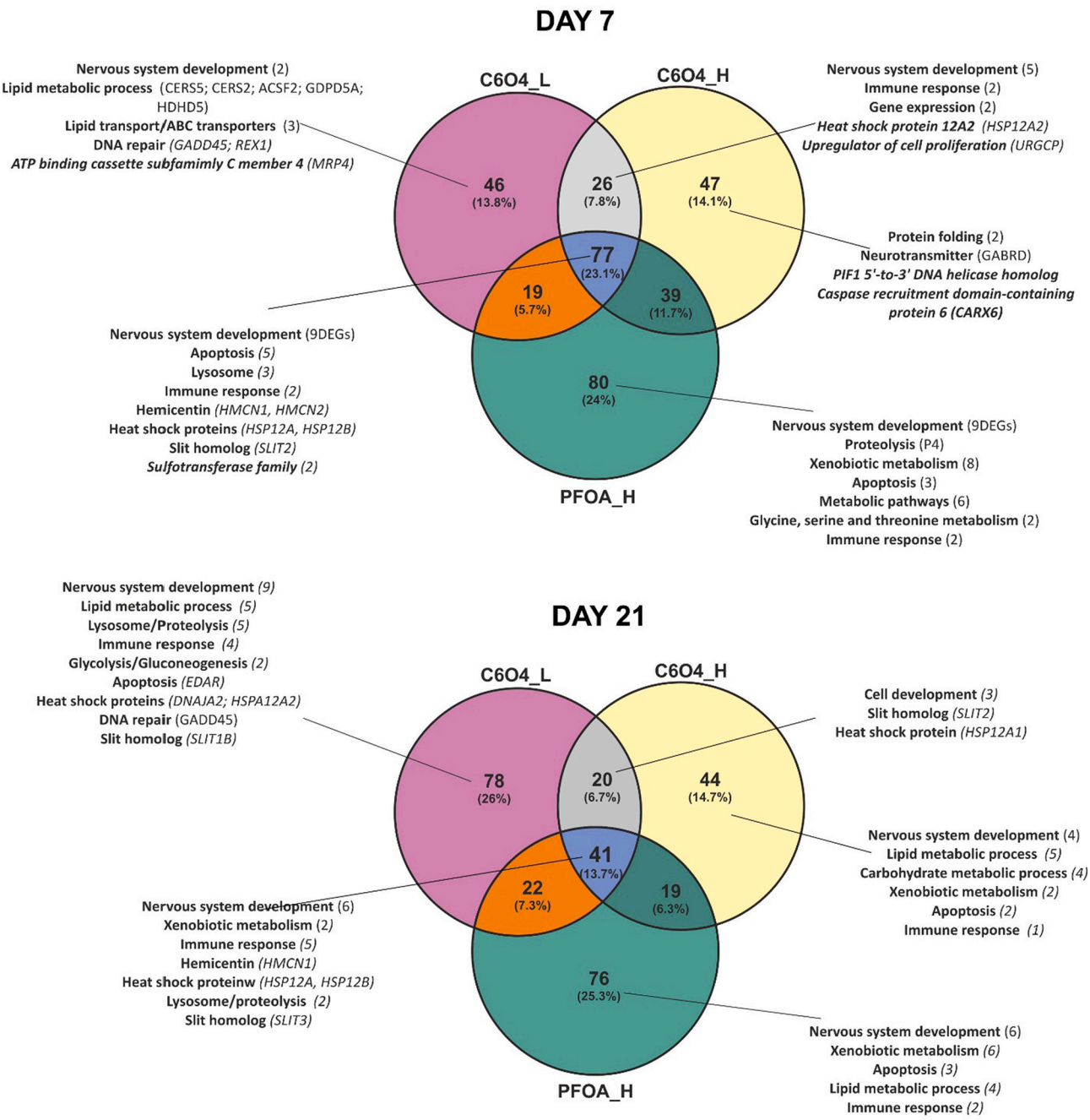

Fig. 3. Venn diagrams reporting the number of differentially expressed genes for each pairwise comparison among control and exposed groups. Gene lists for each treatment was obtained considering the annotated genes through Blastx against zebrafish Ensembl proteins, and *Danio rerio* gene id were considered. Functional annotation and part of differentially expressed genes were also reported referring to genes found differentially expressed uniquely or commonly found differentially expressed among treatments. Full lists of differentially expressed (and corresponding *Danio rerio* gene ID) are reported in Supplementary file S4.

process" in clams exposed to the lowest C6O4 concentration at 7 days, including two up-regulated genes coding for ceramide synthase (*cers5; cers2*). Up-regulated were also glycerophosphodiester phosphodiesterase domain containing 5a (*gdpd5a*) and haloacid dehalogenase like hydrolase domain containing 5 (*hdhd5*) genes, playing a role in sphingolipid metabolism and glycerophospholipid biosynthesis, respectively. Other genes involved in lipid metabolism were differentially expressed in all treatments after 21 days of exposure, among which *cers5* (up-regulated in both C6O4_H and PFOA_H treatments, supplementary File S4). Lastly, among the common response highlighted by Venn diagrams (Fig. 3), several genes coding for heat shock proteins (*hsp12a*; *hsp12b*) were commonly up-regulated in all treatments at both sampling times

The third approach applied to gene expression analyses was based on GSEA related to each treatment/sampling time (adj. p-value < 0.1). The full list of investigated GO_BP and KP is reported in Supplementary Table S6, while the significant pathways are reported in Table 4. First, this analysis confirmed the opposite regulation of several BP and molecular pathways at 7 *vs.* 21 days, suggesting a reduced transcriptional regulation at the end of the experiment. Second, clams exposed to C6O4_L showed a higher number of significant BP/KP compared to C6O4_H and PFOA_H. Among them, up-regulation at 7 days of "glutaminergic synapse", "synapse", "regulation of cell death" and "ABC transporters" should be highlighted. At 21 days an opposite regulation of

Table 4
List of biological processes and KEGG pathways significantly found up-regulated (red) or down-regulated (green) in each treatment/sampling time by GSEA (Adj. p-value < 0.1). The full list of investigated BP and KP are reported in Supplementary File S6.

	DAY 7			DAY 21		
	C6O4_L	C6O4_H	PFOA_H	C6O4_L	C6O4_H	PFOA_H
XENOBIOTIC METABOLISM						
KEGG_ABC_TRANSPORTERS						
DEVELOPMENT						
ORGAN DEVELOPMENT						
STRESS RESPONSE						
GO_ION_TRANSMEMBRANE_TRANSPORT						
GO_RESPONSE_TO_ENDOPLASMIC_RETICULUM_STRESS						
NEUROTRANSMISSION/SYNAPSE						
GO_EXCITATORY_SYNAPSE						
GO_GLUTAMATERGIC_SYNAPSE						
GO_SYNAPSE						
CELL CYCLE/APOPTOSIS/CELL DEATH						
GO_CELL_CYCLE						
GO_REGULATION OF CELL CYLE						
GO_AUTOPHAGIC_CELL_DEATH						
GO_REGULATION_OF_CELL_DEATH						
SIGNALING						
GO_CANONICAL_WNT_SIGNALING_PATHWAY						
KEGG_NOTCH_SIGNALING_PATHWAY						
IMMUNE RESPONSE/INFLAMMATION						
ACTIVATION_OF_IMMUNE_RESPONSE						
DEFENSE_RESPONSE_TO_VIRUS						
GO_DEFENSE_RESPONSE_TO_VIRUS						
GO_INNATE_IMMUNE_RESPONSE						
IMMUNE_RESPONSE						
KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY						
RESPONSE_TO_BACTERIUM						
WOUND_HEALING						

"synapse" and "regulation of cell death" were found, as well as a significant down-regulation of "response to endoplasmic reticulum stress" and "cell cycle". Third, all treatment showed a significant up-regulation of genes involved in immune response in all treatments at 7 days, confirming the results obtained with the enrichment analysis. However, the up-regulation of "innate immune response" was conserved at 21 days only in clams exposed to the lowest C6O4 concentration, while no changes or opposite regulation were found in response to C6O4_H and PFOA_H.

4. Discussion

Despite still little information on ecotoxicological risk of NGPFASs is available, the prompting progressive replacement of long-chain PFAS by short-chain PFAS including C6O4 is based on the assumption that short-chain PFAS are less hazardous (Kjølholt et al., 2015). However, recent studies on short-chain PFAS revealed low-biodegradation in water, bioaccumulation in fish, invertebrates, plants and algae as well as similar acute toxicity to their traditional counterpart long-chain PFAS (Shi et al., 2015; Liu et al., 2014; Chen et al. 2018a; Gebbink et al., 2017; Gomis et al., 2015; Gredelj et al. 2019; Munoz et al., 2019; Wang et al., 2020). Our study, thanks to an interdisciplinary approach, provides the first overview about the possible effects of C6O4 on marine species as compared to the counterpart long-chain PFOA.

4.1. C6O4 might reach high levels in Manila clam haemolimph and soft tissue

The average PFOA concentration detected in control clams can be considered an indicator of the PFOA concentration in the clam of the Lagoon of Venice. Even if the clams have been subject to a partial depuration in clean waters, they still show measurable concentrations of the PFAS uptaken in their environment of origin, which should represent a lower limit of contamination for clams of the Lagoon of Venice. In fact, the PFOA concentration (0.9 ± 0.5 μg/kg ww) is lower than those measured in the Goro Lagoon (Italy) (1.6–5.8 μg/kg ww) (Mazzoni et al. 2016), which receives river water contaminated by fluoropolymer plants discharges (Valsecchi et al., 2015; Rusconi et al., 2015). The PFOA level observed in the Manila clams of this work is similar to PFOA concentration reported in clams from the Aichi and Kumamoto area, a populated and industrial inland sea region of Japan (0.3–1.5 μg/kg ww) (Fujii et al., 2020), whereas it is higher than that reported in clams from Nunavut, a sparsely populated area of Canada with no industrial activity (<0.4 μg/kg ww) (Ostertag et al., 2009). These findings suggest that the PFAS level in the Manila clams collected in the lagoon of Venice could be consider an indicator of the background concentration of areas impacted

by anthropic activities. Similarly, average C6O4 concentration ww (0.19 ± 0.01 μg/kg) can be considered an indicative level of the C6O4 bioaccumulation in the clams of the Lagoon of Venice suggesting that C6O4 is already diffused in the aquatic environment.

The PFOA bioaccumulation factor (BAF) is in line with literature data for aquatic organisms (Valsecchi et al. 2017; RIVM, 2017). Our data suggest a 5 times lower accumulation potential of C604 as compared to PFOA in clam soft tissues. However, considering that some PFAS, including PFOA, have an immunotoxic potential in the marine bivalve *Perna viridis* - probably due to direct and indirect interactions with the haemocyte membrane (Liu et al., 2018) - we also measured PFOA and C6O4 concentrations in the haemolymph. Contrarily to what observed in soft tissues, concentration in haemolymph (at 1 μg/L of contaminants) showed values higher for C6O4 than for PFOA (Table 1), suggesting that C6O4 might reach high levels in specific tissues, potentially triggering similar toxicity to PFOA despite the lower bioaccumulation in clam whole body.

4.2. C6O4 and PFOA may affect the microbial communities in marine species

The digestive gland is the organ with the most important role in nutrient absorption in bivalve species. In this organ, as in vertebrates, relevant host-microbe interactions occur at mucosal interface of epithelia lining the digestive tract. Many symbiotic bacteria in the digestive gland cooperate with the immune system against pathogen and favouring energy and nutrient uptake (Allam and Pales Espinosa, 2016; Green and Barnes, 2010; Meisterhans et al., 2016). The exposure to environmental stressors, including pollutants, may lead to alterations in the gut microbiota, termed dysbiosis, often associated with a variety of pathological conditions including metabolic disorders, inflammation, and tissue degeneration (Carding et al., 2015; Green and Barnes, 2010; Snedeker and Hay, 2012; Yu, 2012; Jin et al., 2017; Takiishi et al., 2017; Milan et al., 2019, 2018; Iori et al., 2020). To date, few studies described the potential of NGPFASs to alter the microbiome of organisms inducing several disorders related to the microbiota activity (Chen et al., 2018b; Liu et al., 2020; Zhou et al. 2020). The present study suggests that significant changes occur in microbial communities of Manila clam's digestive gland following PFOA and C6O4 exposures. Among the most interesting results, the possible spread of *Vibrio* spp. has been observed in response to both C6O4 concentrations. *Vibrio*, a well-known genus of Gram-negative marine bacteria including species pathogenic also for humans, has been widely described among the dominant fraction of bivalve microbiota including Manila clam (Milan et al., 2019, 2018; Vezzulli et al., 2018; Zampieri et al., 2020). Its over-representation following C6O4 exposure is in line with the opportunistic nature of *Vibrio* spp., which are able to spread following stressful environmental conditions (*e.g.* Frydenborg et al., 2014; Green and Barnes, 2010; Li et al., 2019; Vezzulli et al., 2010), and eventually cause mass mortality events in bivalves (Le Roux et al., 2016; Alfaro et al., 2019; King et al., 2019; Milan et al., 2019). Clams exposed to the lowest C6O4 concentration, at 7 days showed also the significant over-representation of the genus *Arcobacter genus*, an emerging opportunistic pathogen frequently reported in unhealthy or moribund marine organisms such as oysters, abalones, and sponges (Tanaka et al., 2004; Fan et al. 2013; Olson et al., 2014; Lokmer and Wegner, 2015). Slight increases in *Vibrio* representation have been also observed in clams exposed to PFOA at 7 days. Despite both *Vibrio* and *Arcobacter* spp. are common members of the core microbiota of several bivalve species, their spread has been already observed following the exposure to chemicals or in bivalves inhabiting polluted areas (Iori et al., 2020; Milan et al., 2018).

All treatments shared over-representation of the genus *Polaribacter* and under-representation of the genera *Methylophaga*, *Profundimonas,* and *Algicola*, suggesting similar microbial dynamics in response to the two investigated compounds (Table 2).

Unexpectedly, at 21 days the most important changes in microbial communities were observed in response to the lowest C6O4 concentration. This result could be explained by possible reduced feeding rates in clams exposed to higher PFOA and C6O4 chemical concentrations, leading to weaker effects in digestive gland microbial composition. However, during controlled exposure no evident differences in excreted pseudofeces or feces were observed in any treatment. A second hypothesis is provided by hormesis possibly occurring at low concentration. Hormetic response, found in all organisms from microbes to animals, is an inverted U-shaped dose–response model, characterized by a strong effect at low doses, but reduce or no effect at high doses (Calabrese and Baldwin, 2001; Calabrese, 2005; Welshons et al., 2006). Despite hormesis responses have been frequently observed in toxicological studies, including in response to PFAS and short-chain PFAS (Cheng et al. 2011; Liu et al. 2020), additional studies are needed to better interpret the results obtained in this study, and in particular, to evaluate the potential changes in feeding rate at different C6O4 concentrations.

One of the most interesting results from microbiota characterization came from the KP analysis, revealing significant functional changes in microbial metabolic functions in all treatments. Among them, pathways related to lipid and fatty acid metabolism were found enhanced in both C6O4_L and PFOA_H exposures (Supplementary Figure S1). Noteworthy, these effects have been also observed in mice gut microbiota exposed to PFOS and to the ultrashort chain PFAS Trifluoromethanesulfonic acid (TFMS) (Lai et al., 2018; Zhou et al. 2020). Our results evidenced also the disruption of several KP related to "Amino acid metabolism" in both C6O4 and PFOA exposed clams. In this regard, a recent study reported that the dietary PFOS exposure perturbed gut metabolism in adult mice, inducing changes in amino acids and butanoate metabolism, all of which are metabolites widely recognized to be associated with inflammation and metabolic functions (Lai et al., 2018). Similarly, zebrafish exposed to the novel PFASs compound sodium *p*-perfluorous nonenoxybenzene sulfonate (OBS), one of the widely used alternatives to PFOS, demonstrated significant changes in the relative gut abundance of the genus *Vibrio*, as well as changes in various amino acids at metabolite level (Wang et al., 2020). Additional similarities in the effects induced at microbial level by PFAS were provided by the disruption of "N-glycan biosynthesis" and "benzoate degradation" in both C6O4 and PFOA exposed clams, two pathways significantly dysregulated also in mice exposed to TFMS (Zhou et al. 2020). Overall, the results here obtained suggest that both C6O4 and PFOA exposure in Manila clam lead to significant dysregulation of lipid/fatty acid and amino acids metabolism. These effects, widely reported in several model and non-model species following PFAS exposure, may also mediate host-molecular and biochemical changes (Yu et al., 2016; Alderete et al., 2019; Lin et al., 2019; Zhang et al., 2020; Chen et al., 2020; Zhou et al., 2020; Alderete et al., 2019).

4.3. Similarities in the Manila clam transcriptional responses to C6O4 and PFOA

Transcriptional changes are considered the primary response to xenobiotic exposure that precedes the effective toxicity. To our knowledge, a unique study investigated the responses of Manila clam to PFAS exposure through a NGS approach, revealing transcriptional changes of several genes involved in immune response, amino acid and lipid metabolism following the exposure for 4 days to high PFOS concentrations (20 mg/L) (Zhang et al., 2020).

Here, the results obtained show a high similarity in the transcriptional responses to both treatments (C6O4 and PFOA), sharing from 39% to the 62% of DEGs (Fig. 3). Among others, pathways related to immune response, apoptosis regulation, nervous system development, lipid metabolism and cell membrane affected by both C6O4 and PFOA exposures, deserve particular attention.

The sub-epithelial tissues along the digestive tract are among the most haemocyte-rich tissues in molluscs. Both PFOA and C6O4 lead to

significant changes of many genes playing key roles in bivalve immune response (Table 3). In addition, considering that the defence mechanism of phagocytosis and spontaneous cytotoxicity relies on effective cell attachment, the enrichment of "Cell adhesion", representing the BP term with the highest number of DEGs in all treatments, should be highlighted. The overall up-regulation of immune-related genes evidenced at 7 days could be in part related to the spread, in the early phase of chemical exposures, of the two opportunistic pathogen genera *Vibrio* and *Arcobacter* described above. However, immune modulations following PFAS exposures have been already widely described in model and non-model species, including clams (Guruge et al., 2006; Dong et al., 2009; Grandjean and Budtz-Jørgensen, 2013; Zhang et al., 2020). Among the most interesting studies, it is worth mentioning the work by Liu and Gin (2018) on *Perna viridis* investigating the potential immunotoxicity of four commonly detected PFAS. Results from this study revealed reduced haemocyte cell viability and suppression of the immune function by up to 50% of normal performance, underlying the immunotoxic potential of PFASs. Additional evidence from the literature suggests that PFAS can cause suppression of genes related to immunity and specially to cell adhesion (Cui et al., 2009; Guruge et al., 2006), supporting the results obtained in the present work. In fact, both C6O4_H and PFOA_H exposed clams showed at 21 days an increased number of down-regulated genes involved in immunity and cell adhesion.

The most interesting hypothesis explaining how PFAS cause immunotoxicity is the possible intercalation of these chemicals in biological membranes, leading to membrane perturbation in cells and cellular organelles that are involved in an organism's innate defence system (Hu et al., 2003; Liu and Gin, 2018). In our study, membrane perturbations in both C6O4 and PFOA exposed clams is suggested by the enrichment of several BP and CC terms such as "plasma membrane", "integral component of plasma membrane" and "glycosphingolipid biosynthesis", as well as by several DEGs involved in "glycerophospholipid biosynthesis" and by the up-regulation of ceramide synthase (*cers5; cers2*) playing a central role in sphingolipid metabolism (Mullen et al., 2012; Šabović et al. 2020). It should be noted that PFAS exposure has been associated with dysregulated lipid metabolism also in humans (e.g. Chen et al., 2020).

Possible effects of PFOA and C6O4 at the level of nervous system development are suggested by the enrichment of the BP "central nervous system development", as well as by several DEGs involved in "nervous system development" (Table 3 and Fig. 3). Additional evidence was provided by the enrichment of "Notch signalling pathway", promoting the proliferative signalling during neurogenesis, and by the disruption of genes encoding members of the *slit* family, involved in neuronal development and differentiation in vertebrates. However, considering that our study was focused on Manila clam's digestive gland, additional studies focused on other tissues are needed to provide a wider perspective on the possible effects of these chemicals on key molecular pathways related to the nervous system.

Just few genes and enriched pathways involving energy metabolism were found modified in all treatments. Among them, particular attention should be deserved to the *adiponectin* (*adipo*) gene, that resulted up-regulated in response to C6O4_L at both sampling times and to PFOA_H at 21 days. The *adipo* gene is associated to fatty acid oxidation, regulation of glucose metabolism, inflammatory processes and anti-apoptosis in vertebrate species (Berg et al., 2002; Tsatsanis et al., 2005; Park et al., 2007). In invertebrate species *adipo* seems to play vital roles in energy metabolism and autophagy (Zhu et al., 2008; Kwak et al., 2013; Chen et al., 2019). In addition, a recent study focused on the oyster *Crassostrea gigas* suggested that *Adiponectin receptor* plays a key role in the immune response of oysters by regulating the expressions of inflammatory cytokines and haemocyte apoptosis (Ge et al., 2020).

While the role of *adipo* in the apoptotic process is not yet clear in invertebrates, the role of programmed cell death in homeostasis maintenance and immune defence have been widely described also in mollusc species (Romero et al., 2015; Nguyen et al., 2019;). Here, a largely overlapping profile of apoptosis regulation was observed in both treatments of experimental clams (Table 3). This profile is also in agreement with the results obtained in other bivalve species exposed to pollutants (Pruski, 2002; Sokolova, 2004; Romero et al., 2011;). In particular, up-regulation of caspases, *birc* and *xiap* genes were reported in mussels and clams in response to a variety of different chemicals (e.g. NSAIDs, heavy metals, and glyphosate among others) (Milan et al., 2016, 2013b; Mezzelani et al., 2018; Iori et al., 2020). The transcriptional changes of several genes involved in apoptosis is likely to be a generic response to chemical/environmental stress rather than a specific mechanism activated in response to PFAS or short-chain PFAS exposure. Similarly, the up-regulation of several genes coding for heat shock proteins (*hsp12a*, *hsp12b*) may represent a generic reaction to chemical stressors (Monari et al., 2011). However, their up-regulation also at lower concentration of C6O4, as well as the dysfunction of the other key molecular pathways here described, represents a warning signal pointing to the potential toxicity of this new short-chain PFAS.

4.4. Manila clam shows unexpected dose-dependent responses to C6O4

While clams exposed to the two concentrations of C6O4 showed similar transcriptional responses in the early phase of chemical exposures, at 21 days a different pattern appeared. The clear separation of clams exposed to the lowest concentration of C6O4, observed also in PCA, can be related to the general slowdown of gene transcription observed at 21 days just in clams exposed to the highest C6O4 and PFOA concentrations (65% and 76% of down-regulated genes, respectively).

Overall, these different transcriptional trends can be explained *i)* by hormesis occurring at low concentration or *ii)* as a response to highest chemical concentrations triggering cells to start a coordinated response to protect themselves from damage through a rapid down-regulation of many genes in a process called Stress-Induced Transcriptional Attenuation (SITA; Aprile-Garcia et al., 2019). Down-regulation of many genes in response to triggers of cellular stress, such as high temperatures, lack of nutrients or infections, is a phenomenon that is poorly understood, although described in many different species such *Drosophila melanogaster*, mouse and human cells (Gasch et al., 2000; Mahat et al., 2016; Duarte et al., 2016). Noteworthy, a recent study that investigated SITA as a consequence of heat shock as a stress model, suggested that protein ubiquitination plays a key role in this transcriptional down-regulation program. In our study, "protein ubiquitination" BP term was enriched just in response to the highest concentrations of C6O4 and PFOA, represented at 7 days by several up-regulated genes including E3 ubiquitin-protein ligase as *trim33* and its homologs *trim24* and *trim28*, thought to be transcriptional corepressors (Agricola et al., 2011).

Among the most evident differences in the transcriptional responses to the two C6O4 concentrations there was also the conserved up-regulation of several KG and BP related to immune response in clams exposed to the lower C6O4 concentration at 21 days (Table 4). This result could be explained by the possibility to keep an "active" gene transcription at lower C6O4 concentration, as well as by stronger host-microbiota interactions that may play a key role in the overall modulation of host-immune responses (Lai et al., 2018; Wang et al., 2020).

4.5. Specific hazards derived by C6O4 and PFOA exposures

While most of the disrupted molecular pathways are common in C6O4 and PFOA exposed clams, specific response to chemical exposure were also observed. Among them, clams exposed to PFOA showed a much higher change in genes involved in xenobiotic metabolism. This is demonstrated by the enriched term "xenobiotic metabolic process", by the up-regulation of several genes belonging to cytochrome P450 superfamily (CYP450), and by the highest number of up-regulated genes belonging to sulfotransferase family (*sult*) detected in PFOA exposed clams at both sampling times. While CYP450 plays a role in phase I drug metabolism, *sult* is a family of phase II detoxification enzymes that are

involved in the homoeostasis of endogenous compounds, as well as in protection against xenobiotics (Negishi et al., 2001). Up- regulation of *sult* in bivalve species was reported in several studies of controlled exposures to chemicals (Guo et al., 2017; Lavado et al., 2006; Milan et al., 2013b) as well in populations inhabiting polluted areas (Milan et al., 2015, 2013a). On the one hand, this result suggests different capacity of clam detoxification enzymes to be induced by C6O4 and PFOA. On the other hand, the marked activation of the detoxification systems following PFOA exposure could be able to limit the effects highlighted in other biological processes described below.

In particular, possible narcotic or neurotoxic effects specific to clams exposed to C6O4 were suggested by the gene expression analysis. First, GSEA revealed significant changes for GO terms related to synapse only in C6O4 treated clams, as "glutaminergic synapse", "synapse" and "excitatory synapse". Second, transcriptional changes of acetylcholinesterase (*ache*) and neuroligin 3 (*nlgn3*) were exclusively reported in clams exposed to the lowest C6O4 concentration. Similarly, gamma-aminobutyric acid receptor subunit theta (*gabrq*), was over expressed in the C6O4_H group at 7 days, whereas the same experimental group showed down-regulation of neuroligin 4 (*nlgn4*) at 21 days. *nlgn3* and *nlgn4* belong to a family of neuronal cell surface proteins exhibiting synaptogenic activity (Chih, 2005; Nam and Chen, 2005). Homologues of mammals NLGN have been identified in the nervous system of many invertebrates suggesting a possible role in synaptic formation (Knight et al., 2011). While GABRQ mediates the fastest inhibitory synaptic transmission in the central nervous system, AChE is an enzyme playing a key role in the termination of synaptic transmission through the breakdown of acetylcholine and of other choline esters that function as neurotransmitters. It was demonstrated that AChE is inhibited by several chemicals such as pesticides and some metals (Gill et al., 1991; Mora et al., 1999; Key et al., 2002; Dailianis et al., 2003), and it was also inhibited in clams inhabiting polluted areas (Matozzo et al., 2005). The literature suggests that some PFAS may be neurotoxic. In particular, relationships between PFAS exposures and neurotransmitter alterations (Dassuncao et al., 2019), neuronal differentiation (Slotkin et al., 2008), synapses (Lee and Viberg, 2013) and potential motor deficit (Chen et al., 2014; Onishchenko et al., 2011) were already described in invertebrate species. Noteworthy, Chen et al. (2018b) recently demonstrated the perturbation of multiple neural signalling processes, including cholinergic, glutamatergic, and GABAergic systems as the result of medaka embryos exposure to environmentally realistic concentrations of perfluorobutane sulfonate (PFBS), an industrial alternative to PFOS (Chen et al., 2018b).

Additional specific transcriptional responses to C6O4_L exposure were the up-regulation of *gadd45*, and the down-regulation at 21 days of the GO "Cell Cycle" and "Cell Cycle regulation". GADD45 belong to a group of regulatory molecules that primarily protect cells to ensure survival under stressful conditions by cell cycle arrest, DNA repair and, ultimately, apoptosis activation (Fornace et al., 1989; Hollander et al., 1999). A role of *gadd45g* in cell-cycle could be through its interaction and inhibition of the kinase activity of the Cdk1/cyclin B1 complex (Vairapandi et al., 2002). Accordingly, the transcriptional down-regulation of genes involved in cell cycle regulation of clams exposed to C6O4_L at 21 days may reflect potential cell cycle arrest mediated by GADD45G as a result of cellular stress.

4.6. Conclusion

Following the need to find valid substitutes, C6O4 was recently introduced as one of the alternatives to PFOA, despite the lack of any scientific evidence on the effects of this new chemical when dispersed into the environment. By combining chemical and molecular analyses, the present study provided the first evidence of possible environmental threats of this new compound. The detection of both C6O4 and PFOA into clam tissues of the Lagoon of Venice, despite the absence of local sources of these chemicals, suggests their potential ability to spread throughout the environment and, consequently, their possible entry into the trophic chain. Molecular analyses suggest that C6O4 may cause significant perturbations to the digestive gland microbiota and alter pathways/cell components at transcriptional level, including immune response, apoptosis regulation, nervous system development, lipid metabolism and cell membrane. In addition, clams exposed to C6O4 showed possible narcotic or neurotoxic effects and reduced activation of genes involved in xenobiotic metabolism. Despite PFOA showed higher accumulation potential than C6O4 in soft tissues, our results suggest that the potential effects in marine organisms following environmental contamination are not reduced by replacing PFOA with C6O4. It appears evident the urgent need of extending our knowledge on C6O4 bioaccumulation in natural populations of marine organisms as well as on the potential ways of uptake and their potential depuration ability. Further studies on the effects of this chemical particularly on edible marine species will shed more light not only on the possible environmental hazard but also on risk for human health.

5. Data accessibility

All sequencing files are available in NCBI Sequence Read Archive: BioProject PRJNA663745 (both RNaseq data and microbiota sequencing).

CRediT authorship contribution statement

Ilaria Bernardini: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Valerio Matozzo:** Conceptualization, Investigation, Writing - review & editing, Resources. **Sara Valsecchi:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Luca Peruzza:** Software, Formal analysis. **Giulia Dalla Rovere:** Methodology, Formal analysis. **Stefano Polesello:** Methodology, Resources, Writing - original draft, Supervision. **Silvia Iori:** Investigation. **Maria Gabriella Marin:** Resources. **Jacopo Fabrello:** Investigation. **Maria Ciscato:** Investigation. **Luciano Masiero:** Investigation. **Marco Bonato:** Investigation. **Gianfranco Santovito:** Resources, Supervision. **Luciano Boffo:** Investigation. **Luca Bargelloni:** Conceptualization, Writing - original draft. **Massimo Milan:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Tomaso Patarnello:** Conceptualization, Writing - original draft, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contribution

Tomaso Patarnello, Massimo Milan, Luca Bargelloni and Valerio Matozzo conceived and design the project. Ilaria Bernardini, Silvia Iori, Maria Ciscato, Milan Massimo, Jacopo Fabrello, Luciano Masiero, Luciano Boffo, Maria Gabriella Marin and Valerio Matozzo participated in sampling activities and lab-controlled exposures. Ilaria Bernardii and Giulia Dalla Rovere performed RNA extractions. Sara Valsecchi and Stefano Polesello performed bioaccumulation analyses. Marco Bonato and Gianfranco Santovito performed water chemical analyses. Luca Peruzza, Ilaria Bernardini and Massimo Milan executed all gene expression statistical analyses. Giulia Dalla Rovere executed microbiota statistical analyses. Ilaria Bernardini, Massimo Milan, Tomaso Patarnello, Stefano Polesello and Sara Valsecchi wrote the manuscript. All listed authors edited the manuscript. All authors read, reviewed and approved the manuscript.

Appendix A. Supplementary material

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